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Preparation of antifreeze protein

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PREPARATION OF ANTIFREEZE PROTEIN

Antifreeze proteins (AFPs) are polypeptides produced by a wide range of species, particularly those indigenous to colder climes, which have the ability to inhibit freezing of water and aqueous materials at temperatures below 0°C. In general, it is thought that these proteins function by means of interacting with and inhibiting the growth of ice nuclei, but it is now clear that there are different classes of antifreeze protein which may have different mechanisms of action and different effects. For example, in addition to causing a thermal hysteresis in the freezing/melting behaviour of ice/water systems, some AFPs can influence the shape and size of the crystals of ice formed when freezing does occur, and some inhibit recrystallisation of ice. More recently, it has been suggested that these proteins should instead be known as Ice Structuring Proteins (ISPs) (Clarke, C.J., Buckley, S.L., and Lindner, N., Cryoletters., 23 (2002) 89-92)

These attributes of AFPs mean that they can have profound effects on properties such as the ease of production, the texture and the stability during storage of various frozen preparations and, in recent years, there has been much interest in their possible commercial application, especially in the food industry. For example, control of ice crystal dimensions can lead to particularly favourable textures in frozen confections as described, for example, in WO 98/04146. Improvements in storage properties as a result of inclusion of AFPs in the formulation are described, for example, in WO 98/04147. A review of the occurrence of AFPs and their potential use in the food industry has been presented by Griffith and Vanya Ewart in Biotechnology Advances, vol 13, pp. 375-402 (1995).

To be well suited to such a purpose an AFP needs to combine desirable effects on the frozen materials in which it incorporated, and to be readily prepared on an industrial scale. The latter requirement can be particularly problematic since many of the species in which AFPs have been identified are not readily amenable to commercial harvesting or processing. Some AFPs have been found to be very susceptible to denaturation, which places severe constraints on the isolation methods which can be applied to them. In view of these difficulties, there has been considerable interest in the production of AFPs by means of expressing cloned genes encoding them in more convenient expression hosts, such as microorganisms or easily cultivated and processed plants. For many AFPs, however, this has proved problematic: they are often obtained in poor yield and sometimes lacking in activity.

Among the most potentially useful AFPs which have been identified is a type III AFP from the Ocean Pout, which has been designated HPLC-12. This protein was found to excel in its ability to aid in controlling the shape and size of ice crystals, as described in WO 97/02343. The protein was shown, for example, to outperform the well-known type I AFPs in recrystallisation tests. A further advantageous property identified for type III HPLC-12 was that although it was not produced in substantial amounts in *E. coli*, it could be produced in good yield by expression of a cloned gene encoding its sequence in a transformed yeast, thus providing a potentially much more convenient and economically viable source for industrial scale production than the fish in which the protein naturally occurs.

The present inventors have found, however, that the type III HPLC-12 produced in yeast has a specific activity, as measured in a recrystallisation inhibition assay, that is much lower than that of the protein isolated from the Ocean Pout. They have been able to show that this is a consequence of O-glycosylation of the protein by

the yeast, which does not occur when the native protein is produced by the fish. Surprisingly, only the non-glycosylated species appears to be active. In turn, they have been able to devise a method for substantially suppressing this abnormal glycosylation, and thereby have provided a means for producing the type III HPLC-12 protein which combines the convenience and cost-effectiveness of yeast as a host organism, whilst yielding a product with a potency approaching that of the native protein.

It is already known that expression of genes in heterologous eukaryotic hosts can yield abnormally glycosylated proteins and that, in some cases, this can affect the biological activity of the proteins. This is reviewed, for example, in WO 94/04687. The proteins in these examples are growth factors and, as such, are functionally completely different, however, from the AFPs which are the subject of the present invention. Indeed, such experimental evidence as there is relating to AFPs reveals no clear and consistent pattern with respect to glycosylation and functionality: some AFPs are naturally extensively glycosylated. For example, DeVries et al (Science 172 (1971) 1152), reported that the activity of an AFP found in northern cods and Antarctic notothenioids loses its activity if the pendant disaccharides are removed. In other cases, however, the glycosylation that occurs in nature has been shown not to be important for the AFP activity. For example, Worrall et al (Science 282 (1998)115-117), found that when a naturally glycosylated AFP from carrots was produced without its surface glycans, its recrystallisation inhibition activity was unaffected. A similar lack of dependence on glycosylation, even though it is naturally present, for AFP activity has been noted by the present inventors in the case of a heterologously expressed AFP from rye grass. Thus there is no clear indication of a general link between glycosylation and activity among AFPs. Furthermore, it has been found that increasing the molecular weight of type III AFP or attachment of fusion proteins does not decrease its AFP activity.

Indeed, the evidence suggests that an increase in molecular weight leads to an increase in activity (DeLuca, C. I., Comley, R., and Davies, P. L, Biophysical Journal 74 (1998) 1502-1508.).

Many other AFPs, including type III HPLC-12, are naturally devoid of any pendant glycans and, for these, the issue of abnormal glycosylation has not previously arisen. Although several such proteins have been produced by expression of cloned genes in a heterologous host, this host has hitherto always been a bacterial one in which glycosylation does not, in any case, occur. Thus there is no teaching in the literature which might lead one to anticipate any effect of abnormal glycosylation when expression takes place in a eukaryotic host such as a yeast.

Most secreted proteins of fungi such as *Saccharomycces cerevisiae* are glycosylated during passage through the endoplasmaic reticulum and Golgi. O-linked glycosylation results in the addition of linear chains of four or five mannosyl residues to the hydroxyl groups of serine and/or threonine side chains on the protein surface. When expression of heterologous proteins is carried out in these species, proteins directed to be secreted are typically glycosylated by the same mechanisms. If the gene expressed was not itself originally derived from a fungus, it is likely that the glycans thereby attached at the surface of the expressed protein will not be at the same positions nor have the same structure as those, if any, which are attached when the protein is produced naturally.

Protein glycosylation involves a large number of enzymes and deficiencies in one or more of these can potentially alter the pattern of glycosylation. A loss of activity of the enzyme responsible for transferring the first sugar residue onto a protein could potentially prevent any glycosylation. Accordingly, use of such a protein mannosyl transferase (pmt)-deficient mutant yeast strain has been suggested as a way to overcome the problem of

abnormal glycosylation of heterologously expressed proteins (WO 94/04687). However the situation is complicated by the fact that there is not just one enzyme with this function but several, with different protein-specificities. For example, in a review on protein O-mannosylation, Strahl-Bolsinger *et al* (Biochimica et Biophysica Acta 1426 (1999) 297-307) noted that of ten O-glycosylated proteins studied, six are glycosylated in yeast by the enzymes Pmt1 and Pmt2, while the other four show a decrease or lack of O-mannosylation exclusively in strains where the activity of the enzyme Pmt4 has been abolished. None of the analysed proteins was seen to be hypoglycosylated in another class of mutant, in which the activity of enzyme Pmt3 is lost, however this mutation did result in reduced O-mannosylation of chitinase in the genetic background of a *pmt1pmt2* double mutation. No correlations between mannosylation specificity and any sequence or structural features of the protein substrate have been identified, so it is not possible to predict which particular transferase enzyme(s) are likely to be responsible for initiating the glycosylation of any particular protein, whether it is a native protein of the glycosylating species, or a foreign protein produced by heterologous expression therein.

Nonetheless, the present inventors have succeeded in identifying specific yeast mutant strains in which the glycosylation of expressed type III HPLC-12 is substantially suppressed, leading to a marked increase in specific AFP activity. Other mutants, in which the glycosylation of other proteins has been found to be suppressed, were ineffective in the case of type III HPLC-12 .

Thus what is provided by the present invention includes both a specific method suitable for production of type III HPLC-12 in yeast, which leads to a marked improvement in specific activity, and a general method for finding conditions in which this goal can be achieved, which can readily be extended to other mutant groups and other expression host species.

Disclosure of the Invention

This invention is founded upon the discovery that when the antifreeze protein type III HPLC-12 is prepared by expression of the cloned gene in a yeast, a substantial proportion of the secreted protein product has been glycosylated. Such glycosylation is not present in the native protein, and recrystallisation inhibition assays on the separated glycosylated and unglycosylated fractions showed, surprisingly, that the glycosylation effectively abolished the AFP activity of the protein.

In view of this surprising finding, the inventors have gone on to devise a method for increasing the specific activity of the type III HPLC-12 antifreeze protein, or a functional equivalent thereof, when said protein is prepared by expression in a heterologous fungal species of a gene encoding the protein sequence, by means of reducing the extent of glycosylation of the protein. Preferably, said glycosylation is O-glycosylation, by which is meant attachment of pendant carbohydrate moieties to serine and/ or threonine residues at the protein surface. This is the form of abnormal glycosylation most commonly observed when heterologous genes are expressed in fungal expression systems.

For the purposes of this invention, an antifreeze protein is a protein which has significant ice recrystallisation inhibition properties. The specific activity is a measure, per unit concentration of the dissolved AFP, of the ability of the protein to limit the extent of increase in size of ice crystals as a result of recrystallisation, in a given time. This can be conveniently be measured by means of a modified splat assay, as described in WO 00/53029.

Type III HPLC-12 is the antifreeze protein originally identified in Ocean Pout, described in WO 97/02343, or a polypeptide obtained by expression in a suitable host species of the gene encoding said protein. A gene sequence and a suitable expression system in *Saccharomyces cerevisiae* are described in WO 97/02343.The AFP type III HPLC-12 amino acid sequence is shown in Figure 1.

By functional equivalent is meant any protein whose sequence has at least 80%, more preferably at least 90% sequence identity with the sequence of type III HPLC-12 as disclosed in WO 97/02343, and which exhibits AFP activity. It will be appreciated that additional sequence at the N- or C-terminus, or inserted sequences that are not present in type III HPLC-12 would not be considered for the purposes of calculating the % sequence identity.

The species in which expression is carried out may be any suitable fungal species, encompassing yeasts including (but not limited to) those of the genera *Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Schizosaccharomyces* and the like, and filamentous species including (but not limited to) those of the genera *Aspergillus, Trichoderma, Mucor, Neurospora, Fusarium* and the like. Preferably the species selected is a yeast, most preferably a species of *Saccharomyces* such as *S. cerevisiae*. Abnormal glycosylation has been shown to be a feature of expression of heterologous genes in many of these genera.

A gene encoding type III HPLC-12 or a functional equivalent thereof is any sequence capable of being expressed in order to yield the corresponding protein in any of the species discussed above. It may be a cDNA sequence, a genomic DNA sequence, a hybrid DNA sequence, or a synthetic or semi-synthetic DNA sequence. In order to obtain the protein product from such a gene , it is necessary to transform the host species with a construct incorporating said gene and to provide said gene with appropriate attendant regulatory sequences.

For example, the gene may be cloned into an expression cassette capable of directing expression of the gene in a fungal cell. Methods for achieving this are conventional and well known in the art. A particularly suitable method for expression of a type III HPLC-12 gene in *S. cerevisiae* is documented in WO 97/02343.

In a preferred embodiment the reduction in glycosylation is achieved by means of selecting a strain of the expressing organism which is deficient in the activity of one or more enzymes involved in protein glycosylation. Preferably said enzyme is one involved in the attachment of a sugar residue directly to an amino acid side chain of the protein substrate. More preferred is an enzyme involved in the attachment of a mannosyl residue to the hydroxyl group of a serine or threonine residue of the protein substrate. Because there are typically several such enzymes active in a given fungal strain, it is necessary to select strains deficient in the activity of the specific enzymes that are effective in glycosylating type III HPLC-12 specifically.

In a preferred embodiment, a suitable strain for expression is selected from among glycosylation-deficient mutant strains which have already been identified for the species in which expression is to be carried out. In the case of expression in *S. cerevisiae*, for example, at least four genes have been identified which encode proteins involved in transfer of a mannosyl residue to protein serine or threonine residues, said genes being designated pmt1, pmt2, pmt3 and pmt4. The present inventors were able to investigate mutants in which the activity of one or more of these genes was known to be disrupted. They were thus able to determine that disruption of either pmt1 or pmt2 was effective in reducing the extent of glycosylation of secreted type III HPLC-12. The yield of secreted protein was also found to be affected by the mutations and it was found that the most preferred gene disruption for the purposes of this invention is that of pmt1, since this produces the

highest yield of the unglycosylated, active type III HPLC-12 protein. By contrast, disruption of the gene pmt4 did not have an appreciable effect on the extent of glycosylation or yield.

Accordingly, the invention provides a method for preparing type III HPLC-12, or a functional equivalent thereof, with enhanced specific AFP activity (in comparison to that obtained when the protein is produced in the parent strain) by means of expressing a cloned gene encoding said protein in a strain of *Saccharomyces cerevisiae* deficient in the activity of the enzymes encoded by one or more of the genes pmt1 and pmt2. Preferably, the gene disrupted is pmt1.

The extent of glycosylation of type III HPLC-12 produced by any chosen expressing strain can be readily gauged by methods that are sensitive to the increased molecular weight of the modified protein. For example, the additional mass of the glycan attachments is readily apparent in SDS-PAGE. The application of this method can further be aided by using an antibody preparation, specific for HPLC-12, to perform a Western blot in which the bands due to the AFP (glycosylated and unglycosylated) are specifically detected, with the background of other proteins suppressed. This allows the identification of strains which are effective in suppressing HPLC-12 glycosylation to be identified, for example, without the need to purify the HPLC-12 protein from the medium into which it is secreted. Suitable monoclonal or polyclonal antibodies can readily be prepared by conventional methods. Alternatively, the level of glycosylated and non-glycosylated type III HPLC-12 can be determined using reverse phase HPLC. Furthermore, the HPLC system coupled to mass spectroscopy can be used to investigate specific glycoforms.

Methods such as SDS gel electrophoresis, Western blot, HPLC analysis and HPLC coupled to mass spectroscopy were used by the present inventors to identify the preferred strains of *S. cerevisiae* for type III HPLC-12 production. It could readily be extended to

investigate further mutant strains of this or other species, in order to seek out still more effective expression hosts. Alternatively, an assay based on the recrystallisation inhibition properties of an at least partially purified type III HPLC-12 containing preparation could be used to detect conditions or strains that produce a good yield of active protein.

By identifying the absence of glycosylation as the key criterion in assessing the utility of the product as an antifreeze protein, and by thus providing convenient methods to assay this, the inventors have thus provided a general method for identifying fungal strains suited to the production of active type III HPLC-12 in good yield.

In *S. cerevisiae* there are a number of other genes which have been identified, whose expression products are enzymes involved in later stages of glycosylation and which, in some cases are involved in both O- and N-linked glycosylation (Strahl-Bolsinger *et al* (Biochimica et Biophysica Acta 1426 (1999) 297-307)). Mutants in which these genes have been disrupted could also, in principle be investigated to gauge their suitability for the production of type III HPLC-12 or a functional equivalent.

The method could also readily be extended to other species. In some cases glycosylation-deficient mutants have already been described and in other cases these could be readily identified. For example, WO 94/04687 describes the cloning of a homologue of pmt1 from another yeast, *Kluyveromyces lactis*. This was readily achieved by PCR, using primers designed using sequence information from the *Saccharomyces* gene. The authors go on to describe how sequencing of the *Kluyveromyces lactis* gene would allow a disruption mutant to be constructed for this species. The same strategy could straightforwardly be applied to other fungi. In the light of the finding by the present inventors that the enzymes encoded by pmt1 and pmt2 are most effective in glycosylating type III HPLC-12 in

Saccharomyces, it is probable that proteins homologous to these would be suitable targets for disruption in other species. Once suitable candidate glycosylation mutants are thus acquired or, if necessary, constructed for other fungal species, the same strategy that has been exemplified by the authors for *Saccharomyces* would be applicable to identify the strain in which the best yield of active type III HPLC-12 is obtainable.

Examples

The following examples are provided by way of exemplification only.

Example 1: Determination of recrystallisation inhibition activity of glycosylated and unglycosylated forms of AFP type III HPLC-12 produced by *Saccharomyces cerevisiae*

Enriched fractions of glycosylated and non-glycosylated AFP type III were prepared from fermentation broth.

Fermentation broth containing AFP type III HPLC-12 (15ml) was pipetted into separate conical tubes and 10ml refrigerated ethanol added and mixed for 5 seconds.

Where the pH was lower than 6.0, the pH was corrected with 1M NaOH. The tube was then left on ice for at least 20 minutes or overnight in the freezer before being centrifuged at a temperature of 5°C for 5 minutes at 3000rpm. The supernatant was then decanted into a separate conical tube. The precipitate was washed by adding 40% ethanol at pH 6.0, mixed, placed on ice for at least 20 minutes or overnight in the freezer and then centrifuged as before. Finally, the precipitate was washed with Ultrapure water into a pre-weighed flask, frozen and dried by freeze-drying.

The supernatants from above were decanted into pre-weighed centrifugation bottles and centrifuged again at approx. 4000rpm for a minimum of 20 minutes. The supernatants were transferred into separate round bottom flask for rotary evaporation. The ethanol was removed from the supernatant by rotary evaporation whereby the temperature of the waterbath did not exceed 35°C. Following removal of the ethanol, the aqueous supernatant was transferred to a pre-weighed flask, frozen and the water removed by freeze-drying.

The non-glycosylated and glycosylated AFP type III contents of the resulting freeze dried preparations are shown in Table 1.

Table 1: AFP type III profiles of the freeze dried ethanol precipitate and supernatant.

Material	Non-glycosylated AFP type III as % total protein	Glycosylated AFP type III as % total protein
Freeze dried ethanol supernatant	0.4	41
Freeze dried ethanol precipitate	39.5	19

Whilst the ethanol precipitate still contains some non-glycosylated material, the supernatant is highly enriched in glycosylated material (41% of total protein) compared to the non-glycosylated component (0.4% total protein).

Recrystallisation inhibition (RI) assay

The recrystallisation inhibition activity of glycosylated HPLC 12 was used to determine the activity of the glycosylated and non-glycosylated AFP type III. A sample of 0.0004% protein in 30%

sucrose solution was prepared and measured (3 repeats) in the RI assay. Two control samples were also measured: 30% sucrose solution (i.e. containing no AFP) and 0.0004% non-glycosylated HPLC 12. The results are presented as the change in the mean ice crystal size after undergoing recrystallisation at -6ºC for 1 hour (Table 2).

Table 2: RI inhibition results

Test solution	Growth (microns)
Control sucrose solution	13.0 ± 0.5
Non-glycosylated AFP type III HPLC 12	0.7 ± 0.5
Glycosylated AFP type III HPLC 12	13.4 ± 0.5

The above results show that the non-glycosylated AFP type III HPLC-12 is active as it significantly reduces the amount of growth compared to the control sucrose solution. However, the glycosylated HPLC-12 shows the same growth as the sucrose solution. Therefore, the glycosylated AFP type III HPLC-12 has no effect on the recrystallisation, i.e. it is inactive.

Example 2. Preparation of protein mannosyl transferase (pmt) deficient mutants

The pmt deficient mutants were constructed in *Saccharomyces cerevisiae* VWK18*gal1* (*MATa, leu2, gal1:URA3, ura3*) using the cre/lox gene disruption system described by Guldener et al (Nucleic Acids Res 24(13):2519-24, 1996). DNA fragments with short-flanking homology were generated by PCR using a loxP-Kan-loxP cassette. Correct integration of the cassette was verified by diagnostic PCR and subsequently the Kan gene was removed by expression of the cre recombinase. Correct removal of the cassette resulting in a deleted gene with one remaining loxP site was verified by diagnostic PCR.

The following deletions were constructed:

pmt1(201,2350)::loxP
pmt2(50,2229)::loxP
pmt4(09,2289)::loxP

Example 3. Construction of mutant *Saccharomyces* strains transformed with a gene encoding AFP type III HPLC-12.

To construct a strain capable of efficient, controlled expression of AFP, *pmt* mutants of the host strain *S. cerevisiae* VWK18*gal1* were transformed with multiple copies of an ISP expression cassette derived from pUR3993 plasmid, designed to integrate at the rDNA locus as described by Lopes et al (Gene 1989 Jul 15;79(2):199-206). The rDNA integration cassette was excised from the complete plasmid by digestion with *HpaI* and the approximately 6283 bp fragment introduced into the host strain by trasformation using the lithium acetate method (Gietz R.D. and Woods R.A. Methods Enzymol 2002;350:87-96).

The detail of the rDNA integration cassette and the pUR3993 plasmid are shown in figures 2 (a) and (b) respectively. Transformants were selected for their ability to grow on minimal medium without leucine and screened for production of the AFP during growth on medium containing glucose as carbon source and galactose as inductor.

Example 4. Determination of AFP III HPLC-12 content in fermentation samples

The sample components are separated by reverse phase HPLC using a C18 column and the AFP type III HPLC-12 content determined by UV detection at 214nm by reference to a purified standard.

Apparatus:
AKTA Explorer XT 10
Analytical balance

Various glassware
Various pipettes (minimum class b)

Reagents:

Ultrapure water	Millipore water system
Acetonitrile	HPLC grade, Far UV
Trifluoroacetic acid (TFA)	HPLC grade
Isopropanol	HPLC grade

Preparation of eluents:

Eluent A: 0.05% TFA in Ultrapure water
A volume of 1ml TFA was diluted to two litres with Ultrapure water and mixed.

Preparation of eluent B: 0.05% TFA in acetonitrile
A volume of 0.5ml TFA was diluted to one litre with acetonitrile.

To prepare samples a volume or weight of test material was accurately pipetted/weighed, in triplicate, into separate 50ml volumetric flasks and made to volume with eluent A. Samples were filtered prior to being analysed using the below specified AKTA conditions. Purified non- glycosylated AFP type III was used as the quantification standard. A chromatograph from a typical fermentation sample is shown in figure 3.

The HPLC conditions used for AFP type III analysis were as follows:

Injection type	:	Partial fill
Injection loop	:	100µl
Injection volume	:	50µl
Needle wash	:	20% IPA
Data handling	:	Compaq deskpro computer Windows NT UNICORN V3.21 software UNICORN/A-900 software
Column	:	Vydac Protein/peptide C18 218TP54
Mobile phase line	:	A11, 0.05% TFA in Ultrapure water
	:	B1, 0.05% TFA in acetonitrile
Gradient	:	T0 → T5:100% A11 T5 → T35:100% A11 → 42% A11, 58% B1 T36 → T40: 42% A11, 58% B → 100% B1 T40 → T41.5: 100% B1 → 100% A11 T41.5 → T44: 100% A11
Flow rate	:	1.0ml/min

The AKTA Explorer 10XT chromatography system was fitted with A900 autosampler and a triple wavelength UV detector. Quantification was achieved using the 214nm signal. Other wavelengths such as 254 and 280nm were used for fingerprinting purpose only.

Example 5. Effect of pmt deletion on AFP type III HPLC-12 production in laboratory scale fermenters.

Fermentations were carried out with each pmt mutant in order to determine the effect of the deletion on AFP production compared to the parent strain without any pmt deficiency. Fermentations were carried out as detailed below.

Inoculum preparation

A shake flask containing 50 ml medium consisting of 6.7 g/l YNB (yeast nutrient broth) w/o amino acids (Difco) and 5 g/l glucose·1aq (Avebe) was inoculated with 1.4 ml glycerol stock of the strain and incubated during 48 hours at 30°C at 120 rpm. Subsequently, the inoculum was transferred to a shake flask containing 500 ml medium consisting of 10 g/l Yeast extract (Difco), 20 g/l Bacto Pepton (Difco) and 20 g/l glucose·1aq followed by incubation for 24 hours, 30°C at 120 rpm.

Fed batch fermentations

The 5.5L batch medium consisted of 22 g/kg glucose·1aq, 10 g/kg yeast extract KatG (Ohly), 2.1 g/kg KH_2PO_4, 0.6 g/kg $MgSO_4 \cdot 7H_2O$, 0.4 g/kg Struktol J673 (Schill & Seilacher), 10 g/kg Egli trace metals (a 100x solution of 5.5 g/l $CaCl_2 \cdot 2H_2O$, 3.73 g/l $FeSO_4 \cdot 7H_2O$, 1.4 g/l $MnSO_4 \cdot 1H_2O$, 1.35 g/l $ZnSO_4 \cdot 7H_2O$, 0.4 g/l $CuSO_4 \cdot 5H_2O$, 0.45 g/l $CoCl_2 \cdot 6H_2O$, 0.25 g/l $NaMoO_4 \cdot 2H_2O$, 0.4 g/l H_3BO_3, 0.25 g/l KI, 30 g/l NaEDTA), 1 g/kg Egli vitamins (a 1000x solution of 5 g/l thiamin, 47 g/l meso-inosit, 1.2 g/l pyridoxin, 23 g/l panthotenic acid, 0.05

g/l biotin). The 4L feed medium contained 440 g/kg glucose·1aq, 3 g/l galactose (Duchefa), 25 g/kg yeast extract, 12 g/kg KH_2PO_4, 2.5 g/kg $MgSO_4 \cdot 7H_2O$, 0.8 g/kg Struktol J673, 20 g/kg Egli trace metals, 2 g/kg Egli vitamins.

The fed batch fermentations were performed in standard bioreactors with a working volume of 10 litres. Dissolved oxygen (DO_2) was measured with an Ingold DO_2 electrode (Mettler-Toledo) and controlled by automatic adjustment of the speed of the 6-bladed Rushton impeller to a maximum of 1000 rpm. The pH was measured with an Ingold Impro 3100 gel electrode (Mettler-Toledo) and controlled using 3 M phosphoric acid (Baker) and 12.5 % v/v ammonia (Merck). Temperature was measured by a PT100 electrode and controlled via a cooling jacket and cooling and heating fingers.

The batch phase was started by transferring 500 ml of full grown inoculum to the batch medium. The temperature was maintained at 30°C and airflow at 2 l/min. DO_2 was controlled above 30%, pH at 5.0. When the ethanol signal in the off-gas decreased below 300 ppm the feed phase was started. In the feed phase the temperature was decreased to 21°C and the airflow was set to 6 l/min. The feed rate was applied according to an exponential profile required to maintain a growth rate of 0.06 1/h. The expontial feed continued until the DO_2 level in the fermenter decreased below 15% whereafter the feedrate was maintained linear.

The yield of total AFP after 60 hrs fermentation and the effect of pmt deletion on non-glycosylated AFP productivity was determined using reverse phase HPLC and is shown in table 3.

Table 3: Yields of glycosylated and non-glycosylated AFP type III HPLC-12 determined by reverse phase HPLC

Test organism	Total AFP normalised to parent strain productivity	Non-glycosylated AFP as % total	Fold increase in Non-glycosylated AFP production
parent strain	1.0	23%	1
pmt1 mutant	0.79	67%	2.3
pmt2 mutant	0.61	71%	1.9
pmt4 mutant	0.93	23%	0.93

The data in table 3 shows that deletion of either pmt1 or pmt2 results in an increase in the % of non-glycosylated AFP produced compared to the parent strain. Although the total AFP yield is slightly decreased for both pmt1 and pmt2 mutants, the non-glycosylated yield is increased due to the decreased glycosylation acitivity resulting in a 2.3 fold and 1.9 fold overall increase in non-glycosylated AFP for pmt1 and pmt2 respectively. By contrast, deletion of pmt4 apparently has little or no effect on the % non-glycosylated product produced but does appear to slightly decrease the overall AFP yield. A comparison of the protein profiles for the parent strain and the pmt1 mutant on SDS gel is shown in figure 4. Similar results are obtained from shake flask screening experiments.

The SDS gel clearly shows that the original non-deficient strain contains both glycosylated and non-glycoslylated AFP whilst the pmt1 mutant produces predominantly non-glycosylated AFP. This provides a relatively quick screening method for identifying strains with reduced abiliy to glycosylate the AFP protein.

Example 6. Analysis of Glycosylation patterns of AFP type III HPLC-12 secreted by transformed mutant strain

Investigation of the AFP type III glycoform patterns of the pmt1 mutant compared to the original non-deficient strain was performed by HPLC-MS. The degree of glycosylation and the relative abundance of AFP versus its main glycoforms (AFP with 5-13 mannose units) are compared using the selected ion monitoring (SIM) mass spectrometric responses of their respective most abundant protonated molecular ions. Detection is performed by positive electrospray ionisation mass spectrometry. Separation was achieved by gradient elution using a reversed phase HPLC column as described below.

Apparatus & Reagents:

1050 HPLC module (Hewlett Packard)
Quattro I mass spectrometer (VG, now Micromass)
PRP1 column 4.6 x 250 mm (Hamilton)
Ultrapure water - Millipore-Q water system
Acetonitrile gradient HPLC grade

Preparation of mobile phases for HPLC
A: 1% acetic acid in water
B: 1% acetic acid in 80% aq. acetonitrile

Sample preparation

The samples were diluted 1 in 50 in water (1 g in 50 ml water) and filtered (0.45 µm or smaller syringe filter) prior to analysis.

Equipment conditions

HPLC system	
UV detector	214nm
Injection volume	20 µl (partial loop filling)
Column	Phenomenex Jupiter C18 300A pore, 150 x 2.1 id mm
Mobile phases	Maintained at 30°C A: 1 % acetic acid in water B: 1 % acetic acid in 80% aq.acetonitrile
Flow rate	1 ml/min
Total analysis time	74 minutes

Gradient	Minutes	% B
	0	10
	10	10
	55	65
	57	100
	62	100
	64	10
	74	10

A split rate of 1/5 was applied after the chromatgraphic separation to deliver 200 µl/min to the mass spectrometer

The QuattroI mass spectrometer

Tune page settings (file	Capillary	3.2 V
	Cone	programmed as part of the method
	HV Lens	0.6 V
	Source block temperature	150 C
	Desolvation temperature	150 C
	Multiplier	650 V
	Desolvation gas flow	300 l/h
	Nebuliser gas flow	25 l/h
MS method	Data is collected between 20 and 60 minutes	
	Scan: m/z 100 to 2000 (scan time: 5 sec, interscan delay: 0.1 sec)	
	SIR function 1 (tracking of an AFP III fragment) m/z 284.4 at cone voltage 70 V (marker for the last 3 amino acids at the C-terminal end of AFP III) and m/z 163.01 (oxonium ion, maker for glycopeptides) (span 1Da, dwell time 0.08 sec, inter channel delay 0.02 sec)	
	SIR function 2 (ions for 6 charge state of AFP glycoforms) m/z 1308, 1335, 1362, 1389, 1416, 1443, 1470 and 1497 at cone voltage 20 V (span 1Da, dwell time 0.08 sec, inter channel delay 0.02 sec).	

Figure 5 shows that the non-glycosylated AFP type III yield is increased from 23% to 67% using the pmt1 strain and that although the level of glycosylated product is reduced the glycosylation pattern is similar to that obtained from the non-deficient parent strain.

Claims

1. A method for increasing the specific antifreeze activity of the antifreeze protein (AFP) type III HPLC-12, or a functional equivalent thereof, when said protein is prepared by expression in a heterologous fungal species of a gene encoding the amino acid sequence of the protein , by means of reducing the extent of glycosylation of the protein.

2. A method according to claim 1, wherein the protein glycosylation that is reduced is O-glycosylation.

3. A method according to claim 1 or 2, wherein the specific AFP activity is measured by means of an ice recrystallisation inhibition assay.

4. A method according to any of claims 1 to 3, in which the reduction in protein glycosylation is achieved by means of selecting a strain of the expressing species which is deficient in the activity of one or more enzymes involved in protein glycosylation.

5. A method according to claim 4, in which the reduction in protein glycosylation is achieved by means of selecting a strain of the expressing species which is deficient in the activity of one or more protein mannosyl transferase enzymes.

6. A method according to claim 4 or claim 5 wherein a suitable glycosylation-deficient strain is selected from a range of such strains by analysis of the type III HPLC-12 protein, or functional equivalent thereof, which is produced when a gene encoding said protein is expressed in said strains.

7. A method according to claim 6 whereby the analysis of the HPLC-12 protein, or functional equivalent, is based on an assay of its AFP activity or functionality.

8. A method according to any of claims 1-7 wherein the heterologous fungal species in which the type III HPLC-12 protein, or functional equivalent, is expressed is a yeast.

9. A method according to claim 8 wherein the yeast is *Saccharomyces cerevisiae*.

10. A method according to claim 9 wherein the *Saccharomyces cerevisiae* in which the type III HPLC-12 protein, or functional equivalent, is expressed is a pmt1-deficient mutant strain.

11. A method according to claim 9 wherein the *Saccharomyces cerevisiae* in which the HPLC-12 protein, or functional equivalent, is expressed is a pmt2-deficient mutant strain.

12. A method according to any of claims 1 to 9 wherein the type III HPLC-12 is expressed in a strain of a fungal species which is deficient in an enzyme homologous to the enzyme encoded by the pmt1 gene of *Saccharomyces cerevisiae*.

13. A method according to any of claims 1 to 9 wherein the type III HPLC-12 is expressed in a strain of a fungal species which is deficient in an enzyme homologous to the enzyme encoded by the pmt2 gene of *Saccharomyces cerevisiae*

Abstract

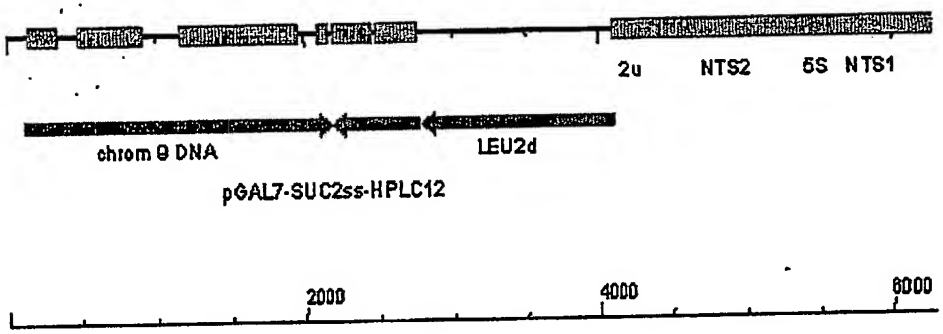
A method is provided for increasing the specific activity of the type III HPLC-12 antifreeze protein, or a functional equivalent thereof, when said protein is prepared by expression in a heterologous fungal species of a gene encoding the protein sequence, by means of reducing the extent of glycosylation of the protein.

Figure 1: Amino acid sequence of AFP type III HPLC-12

1	Asn	Gln	Ala	Ser	Val	Val	Ala	Asn	Gln	Leu	Ile	Pro	Ile	Asn	Thr
16	Ala	Leu	Thr	Leu	Val	Met	Met	Arg	Ser	Glu	Val	Val	Thr	Pro	Val
31	Gly	Ile	Pro	Ala	Glu	Asp	Ile	Pro	Arg	Leu	Val	Ser	Met	Gln	Val
46	Asn	Arg	Ala	Val	Pro	Leu	Gly	Thr	Thr	Leu	Met	Pro	Asp	Met	Val
61	Lys	Gly	Tyr	Pro	Pro	Ala									

(Antifreeze protein type III HPLC-12 is specifically identified by accession number P19614 in the Swiss-Prot protein database)

Figure 2 (a): The rDNA integration cassette



1-126 NTS1 - *Saccharomyces cerevisiae* rDNA non transcribed spacer
127-2186 *Saccharomyces cerevisiae* chromosme IX DNA
114-348 partial orf1= hypothetical protein
485-916 RNAse P subunit
1165-1959 weak similarity. to glucosidase, exo sialidase, mucins
2103-2165 questionable orf
2165-2096 transcriptional activator of sulfur a.a. metabolism
2397-2197- Antifreeze protein type III HPLC12
2457-2398 ISS - *Saccharomyces cerevisiae SUC2* I(Invertase) signal sequence
2775-2486 Pgal7 - *Saccharomyces cerevisiae GAL7* promoter (synthetic)
4009-2801 LEU2d - *Saccharomyces cerevisiae LEU2d*
4100-4413 2u - *Saccharomyces cerevisiae* 2□ plasmid fragment (non functional)
4420-5460 NTS2 - *Saccharomyces cerevisiae* rDNA non transcribed spacer
55461--5581 5S - *Saccharomyces cerevisiae* rDNA 5S RNA
5582-6238 NTS1 - *Saccharomyces cerevisiae* rDNA non transcribed spacer

Figure 2 (b): Map of the pUR 3993 plasmid

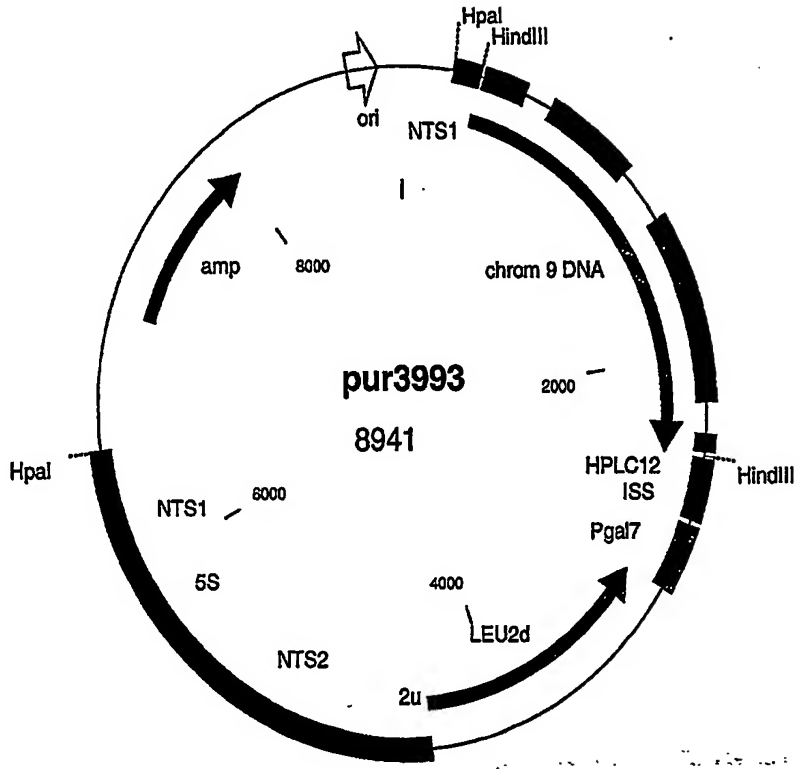


Figure 3: Chromatogram of a typical fermentation sample.

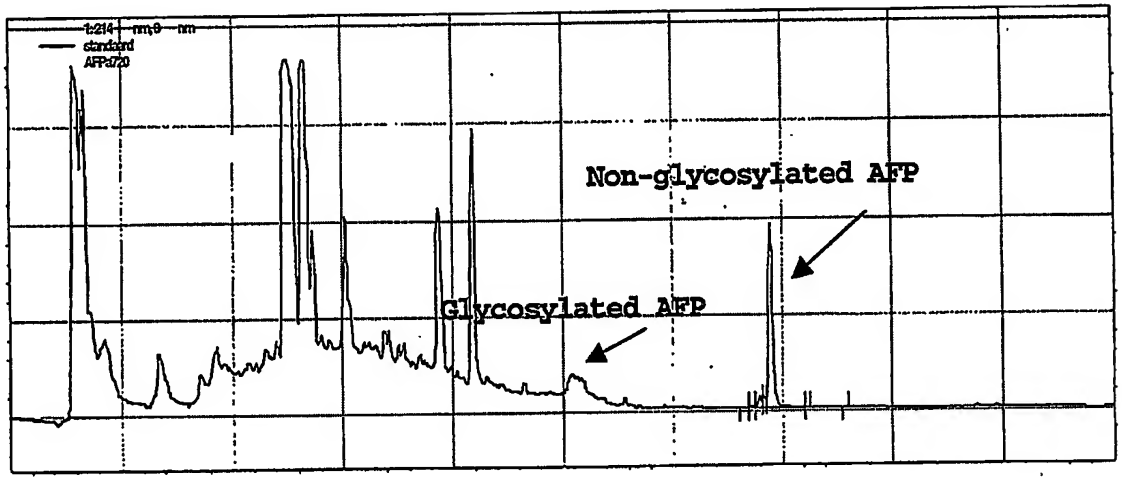


Figure 4: SDS gel showing comparison of parent strain and pmt1 mutant.

Lane 1 = pmt 1 deficient mutant after 60 hrs fermentation

Lane 2 = original non-deficient strain @ 60hrs fermentation

Lane 3 = original non-deficient strain @ 50hrs fermentation

Lane 4 = MW standards

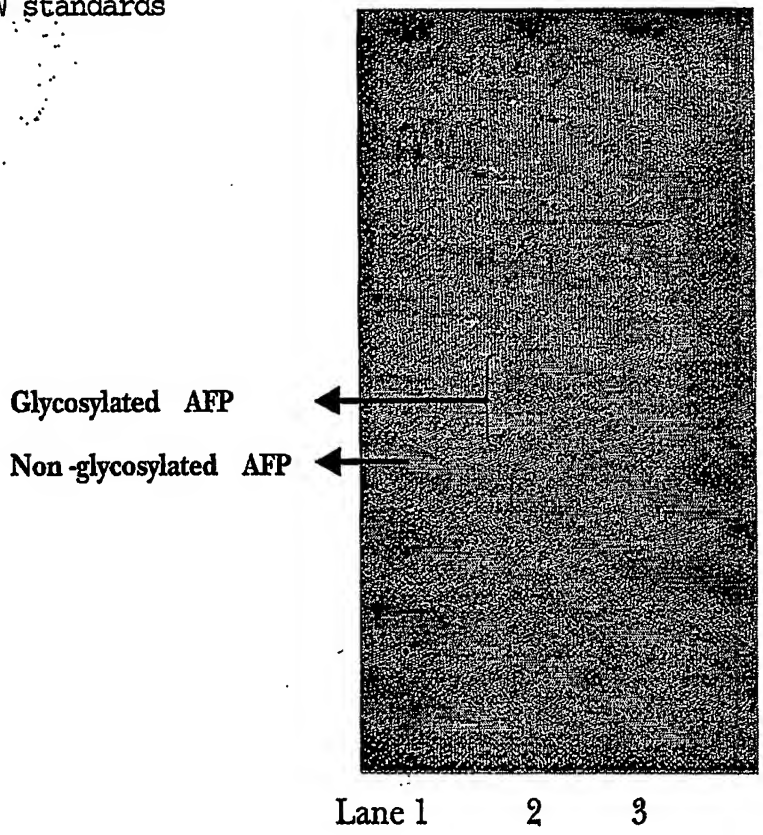


Figure 5: Comparison of the ratio of non-glycosylated and glycosylated AFP type III HPLC-12 produced by original non-deficient parent strain and pmt1 mutant using glucose as carbon source